



Optimizing siRNA Efficacy through Alteration in the Target Cell-Adhesion Substrate Interaction

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ABSTRACT:

Short interfering RNA (siRNA) is a class of nucleotide drugs with a profound potential to improve patient health through its ability to silence the expression of specific genes at the post-transcriptional level. However, the clinical application of siRNA therapeutics remains hindered by a lack of efficient delivery systems that deposit siRNA into the cytoplasm of cells, a step necessary for siRNA's silencing effect. Much research has focused on the development of siRNA delivery agents to overcome this challenge. There are no standard pre-clinical models for testing of siRNA delivery agents, and investigators have chosen to evaluate efficacy in a variety of systems including in vitro tissue culture and animal models. These systems have vastly different cellular microenvironments which may modulate cellular behavior and affect the response of cells to siRNA, thus altering the apparent efficacy of siRNA delivery agents. The substrate on which cells adhere is one aspect of the microenvironment that has been previously shown to alter cellular behavior. In this work, we tested the hypothesis that changing the properties of cellular adhesion substrates can change the apparent efficacy of a siRNA delivery agent. Specifically, we used a commonly employed in vitro cationic lipid siRNA delivery vector and evaluated siRNA silencing efficacy in U251 cells seeded on alginate hydrogel surfaces. These surfaces were synthesized to have systematic variation in integrin ligand arginine-glycine-aspartate (RGD) density and elastic modulus. We found that an eightfold increase in RGD content of the alginate grown substrate increased siRNA knockdown efficacy from $25 \pm 12\%$ to $52 \pm 10\%$, with constant concentrations of siRNA and delivery agent. We found no difference in siRNA mediated knockdown efficacy over the elastic modulus range tested (53-133 kPa). These results indicate that the cell-adhesion substrate interaction can modulate siRNA protein silencing efficacy, a finding important for evaluation of siRNA therapeutics in the in vitro setting.

GLOSSARY

AF488	Alexafluor488
DMEM	Dulbecco's modified Eagles' medium
DS	Degree of substitution
EDTA	Ethylenediaminetetraacetic acid
FACS	fluorescence-activated cell sorting
eGFP	enhanced green fluorescent protein
dsRNA	double stranded RNA
PBS	phosphate buffered saline
RGD	arginine-glycine-aspartate
RISC	RNA-induced silencing complex
RNAi	RNA interference
shRNA	short hairpin RNA
siRNA	short interfering RNA
TCPS	tissue culture polystyrene

INTRODUCTION

This section provides motivation for exploring the interplay between cellular adhesion substrate and short interfering RNA (siRNA) efficacy. It begins with an overview of siRNA, with a focus on the difficult delivery challenges that hinder its translation to the clinic. Subsequently, past work on adhesion substrate microenvironment induced alteration of cellular behavior is explored. In particular, the role of two adhesion substrate properties - integrin ligand availability and mechanical stiffness- are covered in detail. Alginate hydrogels are then introduced in order to justify use of this well validated model extracellular matrix system in the presented experiments. Previous findings exploring the related phenomenon of alginate hydrogel adhesion substrate effects on DNA delivery is discussed in depth. The section concludes with a brief description of this project's aims in relationship to past findings.

A brief history of short interfering RNA (siRNA)

Driven by its immense promise of clinical usefulness, exploration of RNA interference (RNAi) has proceeded quickly in the last two decades. Specifically, this promise lies in the ability of RNAi pathways to mediate potent and specific silencing of a particular protein's expression through modulating the availability of mRNA for translation. Because the morbidity and mortality associated with many diseases results from aberrant protein levels, the ability of RNAi based therapeutics to silence protein expression could alleviate symptoms and progression in a vast array of illnesses[1]. RNAi mechanisms have also found use in other areas where modulation of mRNA levels can alter cellular behavior including tissue engineering and developmental biology[2][3].

Today, RNAi mechanisms are considered 'common knowledge' and widely exploited in biological laboratories. However, observations now attributed to RNAi initially puzzled researchers. For instance, investigators in the early 1990's attempted to enhance the depth of

petunia petal color by introducing colored pigment genes. Instead of yielding more deeply colored flowers, the investigators found the opposite. Specifically, the generated flowers had areas of white on their petals where pigment production was completely inhibited. Similarly unusual, unexplained findings were observed in other plant and animal studies[4][5]. It was not until Fire and Mello's 1998 experiments on *C. Elegans* that an accurate hypothesis for this phenomenon was identified. Specifically, they proposed that short pieces of double-stranded mRNA could operate post-transcriptionally to silence genes[6]. This hypothesis and their findings won them the 2006 Nobel Prize in Medicine, in addition to initiating the rapid development of the RNAi field[5]. With ensuing years, it has become clear that RNAi is an evolutionary ancient mechanism, conserved in many cells, including mammalian ones[7][8]. Via the RNAi pathway, it is possible for cells to down-regulate virtually any type of protein with high specificity and potency[9].

RNAi gene silencing mechanism

Exploration into RNAi mechanisms is ongoing. At the present time, the term RNAi is used to describe two types of post-transcriptional gene silencing: the normally exogenously induced short interfering RNA (siRNA) pathway and the endogenous microRNA pathway[9]. In the siRNA pathway, an RNase III enzyme called Dicer recognizes long, annealed, double stranded RNA (dsRNA) and cleaves this dsRNA into smaller 21-nucleotide double stranded fragments called short interfering RNAs (siRNAs). Subsequently, a protein RNA-induced silencing complex (RISC) containing Argonaute 2 (ARGO2 or EIF2C) recognizes and binds these RNA fragments[10]. The complex then mediates degradation of the siRNA sense strand and employs the retained anti-sense strand to identify mRNA molecules with complementary sequences. Sequence specific mRNA recognition by the anti-sense strand containing RISC

complex leads to cleavage of the mRNA, thus inhibiting downstream translation. This cleavage occurs in a highly stereotyped manner between the mRNA nucleotides paired with positions 10 and 11 on the guiding siRNA anti-sense strand, counting from the 5' end. After cleavage occurs, the siRNA anti-sense strand containing RISC complex is free to initiate another, new sequence specific round of mRNA cleavage. As a result of this repeated process, a sharp attenuation in the protein expression encoded by the cleaved mRNA is observed.

The second microRNA pathway of RNAi works through a similar mechanism of directed mRNA silencing. However, in contrast to the siRNA pathway, the microRNA pathways involve a dsRNA with strands of mostly, but not complete, sequence complementarity, i.e. base pair mismatch is found within these dsRNAs. This results in Dicer cleavage products of short RNA fragments possessing some mismatched base pairs with their target mRNA. Investigations into the mechanisms of action are ongoing, but this low frequency of mismatching results predominantly in RISC complex mediated translational repression as opposed to direct mRNA degradation[11][12].

Therapeutic application of RNAi

Clearly, due to its ability to specifically silence gene expression, RNAi presents an attractive pathway for therapeutic intervention. Most investigations into the exploitation of the RNAi pathway for clinical application have involved two major approaches. The first approach involves delivery of a viral DNA vector designed to encode a specific sequence of mRNA that anneals with itself to form a hairpin loop structure. This mRNA is called a short hairpin RNA (shRNA). With the correct sequence design, the shRNA can be recognized and processed by the Dicer complex into active siRNA or microRNA components. These components can then specifically repress protein expression via the mechanisms described previously. The second

approach involves the direct intracellular delivery of siRNA mimics. In this approach, double stranded 21 nucleotide RNA fragments are used to exploit the RNAi pathway at the step of RISC recognition[9]. In theory, both pathways may lead to effective therapies.

A variety of investigations at all stages are underway utilizing the RNAi mechanism. They include siRNA based treatments for cancers, neurodegenerative conditions and viral diseases, among a variety of different illness[13]. Targets for siRNA include pro-angiogenic factors, chemotherapeutic resistance factors and HIV encoded RNAs[14][15][16]. However, some controversy exists whether these applications are actually acting through RNAi based mechanisms or immune stimulatory mechanisms triggered non-specifically by exogenous nucleic acid exposure[17].

Most would agree that the promise of RNAi based therapeutics has not yet been borne out in the clinic[18][19][20]. The most important challenge facing application of this technology is the development of effective delivery methods for either shRNA encoding DNA plasmids or siRNAs[21][6]. Nucleic acids are highly charged macromolecules that cannot easily pass through hydrophobic lipid bilayers. For example, siRNA must reach the cytoplasm in order to interact with the RISC complex and mediate downstream silencing effects. When introduced into the extracellular milieu, some portion of siRNAs is taken up via membrane bound cellular vesicles called endosomes. However, siRNAs often remain sequestered within these endosomes due to the hydrophobic vesicle membrane perimeter. As a result, they are unable to escape either the endosomal/lysosomal pathway leading to siRNA degradation or the recycling endosomal pathway leading to expulsion of siRNA back to the extracellular space[22]. Although virus derived delivery agents have shown experimental success in overcoming this endosomal entrapment barrier, skepticism regarding viral vector safety remains widespread. This has

resulted in increased focus on non-viral agents, including polymers, as delivery agents for siRNA. Unfortunately, the transfection efficacy of non-viral agents still lags far behind that of viral ones[12][9].

At the present, a substantial amount of work has been undertaken to understand the barriers facing the delivery of hydrophilic biomacromolecules like siRNA. Experimentation has mainly focused on delivery vector design. However, other factors exist that might control delivery effectiveness or the effectiveness of siRNA once delivered. The cellular microenvironment, consisting of the surrounding in which a cell grows, is preeminent among these factors. Furthermore, methods for evaluating siRNA delivery vectors have often in vitro substrates which may not closely resemble physiological microenvironments, possibly resulting in differential delivery vector efficacy when tested in an in vitro versus in vivo setting.

Substantial evidence exists demonstrating that cellular growth environments can significantly alter cellular behavior, including alterations important for biomacromolecule delivery. These behaviors include ligand uptake and cellular proliferation[23]. In the following discussion, the work suggesting that microenvironment can alter cellular behavior, and by extension, the cellular response to biomacromolecular therapies, will be discussed in greater detail.

The substrate on which cells grow influences cellular behavior

Within the *in vivo* environment, the adhesion substrate immediately accessible by the cell consists primarily of extracellular matrix (ECM) components. In both healthy and diseased physiological states, previous work has identified instances of ECM mediated changes in cellular behavior that may have potential downstream effects on siRNA efficacy. For example, ECM can alter cellular proliferation. Such an effect may influence the intracellular concentrations and thus

the silencing potency of siRNA. In addition, ECM and cell interactions can alter cellular responses to extracellular signaling molecules. Because siRNA is also administered to the extracellular space, ECM mediated alterations in cellular behavior may also influence cellular response to siRNA. Because these two properties of proliferation and extracellular signal response illuminate the possible role of adhesion substrate in siRNA delivery efficacy, selected examples from the literature are presented to illustrate adhesion substrate influence on these behaviors in greater detail.

In several different *in vitro* systems, both the density of adhesion moieties and mechanical stiffness of the adhesion substrate have been shown to alter cellular proliferation. Early on, it was observed that the presentation of ECM molecules, like fibronectin, could activate intracellular signaling cascades that induced the G0/G1 transition involved in cellular proliferation[24]. Investigation revealed that these signaling cascades were activated by cell surface receptors called integrins. Integrins bind specific amino acid residues of the ECM, particularly the arg-gly-asp (RGD) amino acid sequence, and compose a large family of heterodimeric structured receptors. The alpha and beta subunits of the heterodimer both possess transmembrane and cytoplasmic domains, allowing integrins to convey extracellular changes into the intracellular space[25]. Evidence has accumulated to suggest that integrin receptor clustering can induce increased cellular proliferation[23]. Thus, it is logical that the increased presence of RGD ligands within an ECM could induce integrin receptor clustering, resulting in increased cellular proliferation. However, availability of integrin receptor ligands is not the only factor controlling proliferation. Specifically, cells cultured on soft ECM gels that do not allow for cell spreading inhibited cell division despite high levels of RGD[26][27]. This suggests a role for mechanics of adhesion substrates in the initiation of integrin mediated cellular

proliferation. It has been now established that both RGD availability and adhesion substrate mechanics play important roles in triggering cellular proliferation. For example, with hepatocytes, it was discovered that while integrin ligands alone are sufficient to promote the G0/G1 transition, entry into the S phase and proliferation required cell spreading- a phenomenon only possible on a substrate of adequate stiffness[28]. Findings demonstrating the importance of both RGD ligand density and adhesion substrate mechanics on the ability of cells to proliferate have been observed in other cell types as well, including pro-osteoblasts and myoblasts[29][30].

A similar wealth of previous data exists to support the role of adhesion substrate in modulating cellular response to secreted ligands. One example where the ECM plays a major in vivo role is via the development of the mammary gland. Here the interplay between the developing epithelium and stroma directs epithelial responsiveness to secreted hormones. Specifically, it has been shown that mammary stroma (of the mammary fat pad) can induce estrogen-responsive progesterone receptor expression. Through this mechanism, the mammary gland stroma stimulates and maintains the estrogen responsiveness of mammary epithelial cells. Similar examples can be found in diseased states[31]. There is accumulating evidence that abnormalities of ECM amount and composition are associated with the development of malignancy. It has been suggested that these alterations may change the behavior of cancerous cells, perhaps enhancing their resistance to chemotherapy and radiotherapy. For instance, ECM components hyaluronan and fibronectin have been found to accentuate chemoresistance in multiple myeloma[32]. These two examples are simply a sampling of the diverse literature supporting a role of the extracellular matrix in controlling cellular response to extracellular molecules. Extracellular matrix moieties have been shown to modulate many other cellular

behaviors including migration, ligand expression and many different internal signaling processes including acting on Rho GTPases and MAPK pathway[33][34][35].

Clearly, a wealth of evidence in diverse models exists to illustrate the role of adhesion substrate in controlling cellular proliferation and cellular response to secreted ligands[30]. Because of the importance of these behaviors in siRNA delivery efficacy, it is logical to theorize that the adhesion substrate may also affect siRNA delivery efficacy via these mechanisms. The goal of this project is to examine this hypothesis.

Alginate hydrogels as controllable cellular adhesion substrates

The proposed investigation on the relationship between adhesion substrate and siRNA delivery efficacy requires a suitable model system. Alginate hydrogels offer a well-validated choice as a model substrate for the proposed experimental questions due to their controllable chemical and mechanical properties[36].

Hydrogels are a vast class of biomaterials with applications spanning clinical, basic science and industrial settings. Broadly speaking, hydrogels are defined as polymeric networks that, due to their hydrophilic nature, adsorb a large quantity of water. As result, they are often soft and flexible, with properties that can mimic human tissues. The polymeric component of a hydrogel may be either synthetic or naturally derived. Alginate is of the latter type, extracted from brown seaweed. Polymers of alginate have a distinctive structure, composed of (1-4)-linked beta-D-mannuronic acid (M units) and alpha-L-guluronic acid (G units). Alginate is a block polymer, which means that alginate polymers are composed of extended stretches of only M units joined to a stretch of only G units. Divalent ions, most often Ca^{2+} , can be used to ionically bind G-blocks between adjacent alginate chains. These ‘divalent bridges’ result in the

solidification of alginate solutions and hydrogel formation[36]. Other methods to form bridges between alginate chains have also been explored, including covalent conjugation[37].

The low cost, relative ease of gel formation and biocompatibility of alginate hydrogels has made them attractive materials for medical applications. However, alginate itself is highly resistant to protein adsorption because of its highly hydrophilic surfaces[38]. As discussed previously, cellular adherence is critical for many cell type's survival and proliferation. Thus, alginate hydrogels alone are not good cellular adhesion substrates[36]. To remedy this, much effort has been undertaken to modify alginate hydrogels with ligands in order to support cellular attachment and thus, normal growth. Most significant among these modifications is the addition of specific cell adhesion molecule RGD peptides[39][29]. Via this approach, aqueous carbodiimide chemistry is used to covalently attach primary amines of RGD peptides to free carboxyl groups on the alginate[29]. RGD modified alginate polymers, in contrast to their non-modified forms, are able to support cell attachment, growth and proliferation. As a result, they have been used in many studies, including those that examine the fundamental properties governing cell/adhesion substrate interactions [29][39][36].

A major advantage of RGD-modified hydrogels for use in this investigation is that they allow for control of both chemical and mechanical properties of interest. In terms of the chemical properties, modifications of the RGD chemical crosslinking procedure allows for modulation of the density of RGD molecules on the alginate surface. Changes in RGD density can alter the number of cellular receptor (integrin) and adhesion substrate (RGD ligand) interactions. As discussed previously, integrins provide a key mechanism by which cells interact with their microenvironment and then translate environmental signals into intracellular biochemical cascades. Thus, the density of RGD ligands on a surface presents a control point for

understanding cell/material interactions[30]. In terms of the mechanical properties, by altering the concentration of Ca^{2+} used in the alginate crosslinking procedure, it is possible to change the stiffness of the gel. Increasing Ca^{2+} crosslinker results in a stiffer gel[29]. As discussed previously, this represents a second important control point for cell/materials interactions, since a variety of studies have shown that adhesion substrate stiffness plays a key role in cellular proliferation, differentiation and other behaviors. In this work, alginate will thus be used as an adhesion substrate that can be manipulated to examine the role of RGD density and stiffness of siRNA delivery efficacy.

Regulation of DNA delivery efficacy by adhesion substrate modification

There is precedent to believe that adhesion substrate can alter siRNA delivery efficacy, because it has been shown to alter the delivery of other nucleic acids. Early experiments have demonstrated that ECM composition can change gene expression mediated by plasmid DNA delivery. Specifically, fibronectin exposure was demonstrated to enhance both cellular proliferation and gene expression of exogenously introduced plasmid DNA [40][41][42]. Because plasmid DNA requires introduction into the nucleus for expression and fibronectin increased proliferation, it was thought that the nuclear membrane breakdown occurring during the increased proliferation mediated the increased gene expression[43][44]. Further work was undertaken by the Mooney Lab to more systematically examine the factors regulating adhesion substrate control of gene expression. In these experiments, RGD alginate hydrogels were used to provide RGD density and stiffness modifiable adhesion substrates. The plasmids used to transfect the cells were delivered as a noncovalent complex with cationic polymer polyethylenamine (PEI). Interestingly, increased RGD density and increased mechanical stiffness of the alginate hydrogels were able to independently increase gene expression of

adherent pre-osteoblast cells. Increases in gene expression correlated with increased cellular proliferation on these adhesion substrates suggesting that again, increased plasmid access to the nucleus due to nuclear membrane breakdown during mitosis may be the mechanism by which increased gene expression occurs[30][23].

Although siRNA is still a nucleic acid, its mechanism of action does not require nuclear entry[9]. Thus, it is of interest to explore how the same adhesion substrate factors that influence DNA gene expression can also alter siRNA expression.

Aims of study

siRNA presents a potentially revolutionary class of clinically effective therapeutics. However, like other biomacromolecule therapeutics, delivery of siRNA remains a major barrier to its use. Although much research has focused on optimizing the delivery vehicles for siRNA, considerably less is known regarding the influence of cellular adhesion substrate on siRNA mediated protein silencing. Based upon the above background evidence, **it is hypothesized that altering the properties of cellular adhesion substrates can alter siRNA delivery efficacy.** Towards evaluation of this hypothesis, alginate cellular adhesion substrates will be generated with alterations in two variables: adhesion substrate RGD ligand density and substrate stiffness. These variables and the range of values for these variables were chosen because they have previously been shown to alter the delivery efficacy other nucleic acid compounds[30]. Subsequently, siRNA mediated knockdown of enhanced Green Fluorescent Protein (eGFP) expression in a stably transfected eGFP U251 (human glioma) cell line seeded on the different alginate substrates will be assessed to determine siRNA delivery efficacy. U251 cells were used because siRNA therapeutics may be a valuable intervention for gliomas which remain without

good treatment options despite being highly invasive tumors with poor prognoses. The progression of this work can be broken in to three major aims.

- 1) **Synthesize and characterize a series of alginate adhesion substrates.** A series of alginate polymers with variations in either the elastic modulus or the extent of RGD integrin ligand density will be synthesized. Values for the elastic modulus and RGD density will approximate those used in previous studies examining the effect of substrate on other types of nucleic acid delivery[30]. Subsequently, these adhesion substrates will be characterized prior to cellular seeding.
- 2) **Investigate the growth and behavior of cells grown on the alginate adhesion substrates synthesized.** Differences in number and surface area of cells grown on the substrates generated in Aim 1 may alter the ability of siRNA to be endocytosed and delivered to the cytoplasm. Thus, prior to determining siRNA knockdown efficacy, analysis of the cellular properties that could alter siRNA delivery will be examined.
- 3) **Evaluate the siRNA delivery efficacy in U251 cells cultured on the alginate adhesion substrates.** To determine the ability of adhesion substrate to alter non-viral siRNA delivery, the efficacy of targeted fluorescent protein knockdown after siRNA administration for cells grown on varying alginate adhesion substrates will be evaluated. Uptake of fluorescently labeled siRNA on different substrates will also be examined to further explore any changes in siRNA knockdown efficacy.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagles' medium (DMEM), fetal bovine serum, penicillin, streptomycin, Lipofectamine 2000, Trypsin/Ethylenediaminetetraacetic acid (EDTA) and Optimem I medium were purchased from Invitrogen (Carlsbad, CA). Geneticin, 2-(N-morpholino)ethanesulfonic acid (MES) buffer, activated charcoal, and EDTA were purchased from Sigma Aldrich (St. Louis, MO). *N*-Hydroxysulfosuccinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were obtained from Pierce Chemical (Rockford, IL). MWCO 2500 Dialysis Membranes were obtained from Fisher Scientific (Pittsburg, PA). Ultrapure MVG alginate was purchased from FMC Biopolymer (Princeton, NJ). RGD peptide G4RGDASSKY-OH was supplied by Peptides International (Louisville, KY). SiRNA was obtained from Integrated DNA Technologies (Coralville, IA). Alexafluor488 labeled siRNA was purchased from Qiagen (Valencia, CA).

Alginate hydrogel synthesis

The synthesis and mechanical characterization of RGD-modified alginate hydrogels was performed according to a previously described procedure [29][45]. Briefly, alginate polymers were covalently conjugated to RGD peptides via EDC/SulfoNHS coupling to yield differing numbers of RGD peptide per alginate polymer (degree of substitution). For example, a gel with an average of two RGD ligands per alginate macromolecule via a previously validated synthesis scheme was designated DS 2[29]. Calcium crosslinking was employed to form hydrogels from RGD-modified alginate. Subsequently, the elastic moduli of gels were adjusted by controlling the concentration of calcium used to form the gels. In this study, four categories of ionically crosslinked alginate hydrogels were generated for use as cellular adhesion substrates, summarized in Table 1.

Cell Culture

Yong Choi and John Park (NINDS, Bethesda, MD) generously provided human malignant glioma U251 cells stably expressing enhanced green fluorescent protein (eGFP) via electroporation transfection with the pEGFP-c1 cDNA plasmid. Limited dilution cloning was undertaken to yield a narrow range of eGFP expression level in clonal populations of eGFP expressing U251 cells. DMEM supplemented with 10% fetal bovine serum, 100 µg/mL penicillin, 100 IU streptomycin and 1 mg/mL geneticin was used as cellular media. The geneticin was added to maintain eGFP expression via the pEGFP-c1 plasmid's geneticin resistance cassette. Cells were passaged biweekly with Trypsin/EDTA, and maintained in a 37°C humidified, 5% CO₂ incubator.

Quantification of cell growth, surface area and numbers

Four types of alginate gels were prepared with systematic variation in RGD density and elastic modulus as described in Table 1. Gels were formed with a 1 mm thickness and 10 mm diameter. Prior to cell seeding, the gels were incubated in 0.5 mL of complete DMEM growth medium overnight in a 37°C humidified, 5% CO₂ incubator. A single gel was then transferred into one well of a 24-well plate. Subsequently, 6×10^4 cells/0.5 mL of growth media was added to each well to seed the gels. Fluorescent imaging was performed with a Nikon Eclipse E800 fluorescent microscope and Nikon camera attachment. Analysis of surface area distributions and cell number recorded in the fluorescent images was done with ImageJ (NIH, Bethesda, MD).

Evaluation of siRNA-mediated eGFP knockdown

Clonal eGFP U251 cells were cultured on alginate gels as described in the previous section, with the exception that media used was antibiotic free DMEM supplemented with 10% fetal bovine serum. Cells were allowed to incubate overnight in a 37°C humidified, 5%

CO₂ incubator prior to the addition of siRNA complexed to Lipofectamine 2000 at various concentrations to each well of the 24 well plate. The siRNA sequence for silencing eGFP is as follows: sense-GCAGCACGACUUCUUCAAGTT, antisense-CUUGAAGAAGUCGUGCUGCTT. A scrambled siRNA control has the following sequence: sense-GUGACAUCGCGACCACUUATT, anti-sense-UAAGUGGUCGCGAUGUCACTT (Integrated DNA Technologies, Coralville, IA). Non-siRNA containing medium within each well was aspirated, and siRNA-Lipofectamine 2000 complexes suspended in 100 µL of OptiMEM I were added per well. The complexes were incubated with the cells on the alginate hydrogels for 4 hours prior to removal of the complex containing media and replacement with 500 µL of complete growth DMEM medium. Cells growing on the alginate gels were then allowed to incubate for a further 72 hours at 37°C and 5% CO₂. After completion of this incubation time, gels and their adherent cells were transferred from their wells into 1 mL of 50 mM EDTA in phosphate buffered saline (PBS) for 15 min to allow the alginate gels to dissolve. The remaining cells were pelleted and resuspended in 300 µL D-PBS. The suspended cells were then filtered through a cell strainer into a fluorescence-activated cell sorting (FACS) tubes for use with the LSR Fontessa (BD software via forward scatter/side scatter gating (FSC/SSC) to isolate viable cells. Further analysis of fluorescent expression was performed with FlowJo V.6 software (TreeStar Inc., Ashland, OR).

Evaluation of siRNA uptake

Fluorescent AlexaFluor488 labeled siRNA was used to assess siRNA uptake. The procedure for siRNA uptake was similar to that described for the eGFP silencing siRNA and scrambled siRNA control. However, FACS analysis was done earlier to evaluate for uptake directly after wash-out of the siRNA-Lipofectamine 2000 complexes on day 1 post-seeding of 6

$\times 10^4$ cells/0.5 mL of growth media. Concentration of AlexaFluor488 labeled siRNA was held constant at 50 nM for uptake experiments. Uptake was determined by comparing the mean fluorescent intensity at 488 nm wavelength for cells receiving AlexaFluor488 labeled siRNA and untreated control cells.

Statistical analysis

SPSS 17.0 (SPSS, Chicago, IL) was used to assess for significance via one-way analysis of variance (ANOVA) with post-hoc Scheffe test. Student t-tests were done with Excel 2007 (Microsoft, Redmond, WA).

RESULTS

Synthesis and characterization of alginate hydrogels

Alginate hydrogels were synthesized with systematic variation in RGD peptide density and elastic modulus as shown in Table 1. There were two levels of RGD density achieved via covalent conjugation to yield a ratio of RGD ligand to alginate polymer of either 2 or 16 (referred to subsequently as the degree of substitution or DS). An example of a typical hydrogel appearance is shown in Figure 1.

These gels maintained their mechanical stiffness over the time course of experiments used in subsequent studies, as shown in Figure 2A. At day four of incubation, the two gels types synthesized with 50 mM and 100 mM Ca^{++} had elastic moduli of 53 ± 23 kPa and 133 ± 31 kPa, respectively. Covalent modification did not alter the mechanical properties of the hydrogel, as shown in Figure 2B.

eGFP U251 cell growth characteristics on alginate substrates

The growth of U251 cells on the alginate adhesion substrates was characterized in terms of cell morphology, proliferation and surface area. Alginate hydrogel substrates used had either DS 2 or DS 16, with an elastic modulus of either 53 or 133 kPa. Cells adhered to all four categories of gel without dramatic variations in cellular morphology (Figure 3). However, there were a higher number of cells grown on alginate substrate had an elongated shape compared to those grown on tissue culture polystyrene (TCPS) as seen in Figure 3.

There was an initial significant difference between the mean surface area of cells grown on the 53 kPa DS 2 alginate gel when compared to 133 kPa DS 16 alginate gel on day 1 post-seeding. Average surface areas for the DS 2 alginate gels with elastic moduli of 53 kPa and 133 kPa were 340 and $314 \mu\text{m}^2$, respectively. Cells adherent to the DS 16 substrates possessed slightly larger average surface areas of 388 and $432 \mu\text{m}^2$ for 53 kPa and 133 kPa, respectively.

Finally, TCPS adherent cells had the largest average surface area of $519 \mu\text{m}^2$. There were no significant differences in cellular surface area for cells grown on the four different alginate substrates the fourth day after seeding, as seen in Figure 4.

Cell number was also measured for cells growing on each of the alginate adhesion substrates. Total cell number was greatest for the alginate gels with the highest degree of substitution or RGD density, as seen in Figure 5A. However, after normalization by the number of cells present at day 1 after seeding, there was no difference in cell number amongst substrates, as seen in Figure 5B.

siRNA gene silencing efficacy on alginate substrates of varying RGD densities and elastic moduli

The effect of variations in alginate hydrogel substrate RGD density and elastic modulus on siRNA silencing efficacy in eGFP expressing U251 cells was assessed via quantification of fluorescence intensity. On the TCPS control surface, U251 cells exposed to 8.25 nM of siRNA designed to specifically silence eGFP complexed with Lipofectamine 2000 had a $44 \pm 3\%$ decrease in eGFP expression, as seen in Figure 6. However, the same cells grown on alginate adhesion substrates did not show knockdown efficacy of siRNA at this concentration, also seen in Figure 6. A scrambled siRNA control showed no significant eGFP expression knockdown for U251 cells grown on TCPS under the same conditions, as seen in Figure 7.

At a higher concentration of 33 nM siRNA, the knockdown effect on cells adherent to TCPS resulted in a higher knockdown effect of $80 \pm 2\%$, as seen in Figure 6. This effect was predominately sequence specific because a scrambled siRNA control demonstrated only a 9% knockdown in eGFP expression, as seen in Figure 7. At this higher concentration of siRNA, there was a significant siRNA knockdown effect for cells adherent on the alginate substrates, although the amount of knockdown effect was less than for the TCPS substrate. Normalization

the knockdown percentage for TCPS and the different alginate substrates by cell surface area one day after seeding eliminated the difference in siRNA knockdown efficacy between TCPS and some, but not all, of alginate substrates.

The gel with the highest mechanical rigidity of 133 kPa and highest RGD content of DS 16 showed the highest amount of siRNA mediated knockdown. eGFP expressing cells grown on these alginate gels showed a $52 \pm 12\%$ knockdown of eGFP expression when exposed to 33 nM of siRNA. The softer gel with 53 kPa elastic modulus and the same RGD content with DS 16 demonstrated a similar $47 \pm 14\%$ knockdown effect.

Reducing the DS from 16 to 2 while keeping the elastic modulus high at 133 kPa resulted in a significant decrease in knockdown efficacy. This eightfold decrease in RGD density reduced the knockdown efficacy of the siRNA by 52% to $25 \pm 10\%$. The softer gel with elastic modulus of 53 kPa and a low RGD density with DS 2 showed the lowest knockdown efficacy of $34 \pm 6\%$.

Quantification of siRNA uptake on different alginate substrates

Cells grown on the different alginate substrates were also evaluated for differences in siRNA uptake. The increase in fluorescence signal from non eGFP expressing U251 cells exposed to fluorescently labeled (AlexaFluor 488) siRNA at one day after seeding was highest for cells grown on TCPS, as seen in Figure 8A. Among the alginate gels, the cells growing on the gels with the highest RGD density had the greatest siRNA uptake. Relative measurements of siRNA uptake were used to normalize the increased silencing efficacy of cells grown on the alginate substrates. Normalization by siRNA uptake eliminated the significant increase in siRNA silencing efficacy seen for between the 133 kPa/DS 2 gel and the 133 kPa/DS 16 gel, as seen in Figure 8B. There was no significant difference in uptake between cells growing on the 133 kPa gels versus the 53 kPa gels when the RGD density was held constant.

DISCUSSION

Synthesis and characterization of alginate hydrogels

Prior studies have shown that calcium crosslinked alginate hydrogels can decrease in stiffness over time. This occurs as the crosslinking calcium ions that hold together the guluronic acid (G) blocks of alginate polymers exchange with monovalent ions from the incubating media[46]. Because one of the goals of the presented experiments was to evaluate the siRNA silencing ability on alginate gels with varying mechanical stiffness, it was important to ensure that the mechanical stiffness of the alginate gels remained constant over the time course of subsequent experiments. Determination of the elastic modulus over the maximum time course of four days used in these studies showed that stiffness of the gels did not vary significantly over time, as seen in Figure 2A. Furthermore, covalent modification of alginate polymers with RGD did not appreciably alter the mechanical properties of hydrogels made with these modified alginates, as seen in Figure 2B.

eGFP U251 cell growth characteristics on alginate substrates

Hydrogels formed from unmodified alginate polymers do not allow for mammalian cell adhesion, and modification of the alginate with RGD is one way to promote cellular adhesion[47]. However, there is often a cell type dependent threshold limit of RGD grafting density, below which a particular cell type does not adhere to the alginate hydrogels[47][48]. Thus, it was of interest to characterize U251 cell morphology, proliferation and surface area on the alginate hydrogels prior to evaluating siRNA knockdown efficacy.

Cells were able to adhere to gels of both DS 2 and DS 16 RGD densities, as seen in Figure 3. General observations of the eGFP expressing U251 cells' morphology did not appreciably differ between the different alginate substrates. However, the cells grown on the alginate gels did have a more elongated appearance compared to cells grown on TCPS, as seen

in Figure 3. This may be due to a higher density of adsorbed adhesion molecules or stiffer substrate of the TCPS compared to the alginate gels resulting in the more flattened and spread out morphology of cells grown on TCPS.

Qualitative observations of cell shape were followed up by quantitative evaluation of cell surface area for each of the four alginate gel types and TCPS. Cell surface area was measured because theoretically, variations in cell surface area might change the opportunities for siRNA particle internalization and thus, the efficacy of siRNA knockdown. However, there were few differences in cell surface area among the different alginate growth substrates at day 1 after seeding. The 53 kPa and DS 2 alginate gel had a significantly smaller surface area compared to the 133 kPa and DS 16 alginate gel. In contrast, the surface area of cells grown on the TCPS substrate was significantly larger than any of the alginate substrates, supporting the observation of less elongated and more spread out cells on the TCPS substrates.

Previous studies have shown that substrates permitting increased cellular proliferation facilitate increased delivery efficacy of DNA/PEI complexes[49][30]. It is possible that a similar effect might occur for siRNA delivery, hence measurements of the U251 cellular proliferation on the different alginate growth substrates was estimated by counting cell number at different time points post-seeding. Although cell number does conflate rates of cell proliferation and death, during the time course studied, cells grown on the substrates were subconfluent and supplied adequate nutrients making it unlikely that cell death was a substantial contributor to the total cell counts.

Although the total cell number was highest at day four post-seeding on the gels with the highest RGD density (DS 16), normalization to the number of cell present on the first day post seeding eliminated the significant difference in cell number for the higher RGD density

substrates. This supports the conclusion that although there might be different numbers of cells initially adherent to the different substrates, cell proliferation was not necessarily increased by differences in substrate properties.

These findings are in contrast to observation in other work where cells have responded to increased growth substrate stiffness and RGD density with increased rates of proliferation. However, these experiments were done in myoblasts and hepatocytes, so it is possible that the presented experiments differences from previous findings are due to a difference in cell type[39][47][48]. In particular, the U251 cell type is derived from human glioma tissue, and as a neoplastic cell line may be under reduced proliferative control by its adhesion substrate. Alternatively, the U251 cell line may be responsive to a different range of elastic moduli of the substrate compared with myoblasts and hepatocytes.

siRNA gene silencing efficacy on alginate substrates of varying RGD densities and elastic moduli

After characterizing the growth characteristics of U251 cells on the various alginate substrates, the effect of these substrates on siRNA gene silencing efficacy was tested. For these experiments, it was presumed that eGFP protein levels reflected eGFP mRNA levels, and that effective siRNA silencing would decrease both protein and mRNA amounts even though only the protein levels were examined.

The siRNA used was verified for sequence specificity by testing eGFP expression for cells exposed to both sequence-specific siRNA against eGFP and a scrambled siRNA control, as seen in Figure 7. The sequence specific siRNA was then used to evaluate for siRNA mediated knockdown on the different growth substrates.

Results from the siRNA experiments presented in Figure 6 showed the range of elastic moduli tested (53-133 kPa) did not significantly impact the efficacy of siRNA knockdown in

U251 cells. This finding contrasts with previous results demonstrating a change in DNA delivery measured by exogenously induced DNA expression in preosteoblasts dependent on the stiffness of alginate growth substrates[30]. The presented experiments cannot definitively identify the mechanistic reason for a lack of this effect in present experiments. It is possible that differences in cell lines can result in differences in the range of elastic moduli that elicit a functional response. Most organs and tissues have a modulus range from 0.1 to 100 kPa, and the alginate substrates used in the presented experiments have elastic moduli that overlap this range. However, the U251 cells used may require further testing at an increased range of substrate stiffness to reveal changes in siRNA efficacy because they may not be responding to changes in adhesion substrates over the range of 53-133 kPa. As discussed previously with regard to proliferation patterns on the different substrates, it is also possible that U251 cell line's origin from human neoplastic tissue may make these cancer cells less responsive to extracellular matrix cues than non-neoplastic cell lines.

The TCPS substrates showed the highest efficacy of siRNA knockdown when used as a growth substrate. Perhaps these substrates' highest elastic moduli resulted in the increased silencing efficacy. Yet, TCPS has a different composition and surface character than alginate gels, and thus, it is impossible to exclude other properties of TCPS besides stiffness that may be altering siRNA efficacy.

Unlike alteration in elastic modulus, RGD density of the alginate substrates did not significantly alter the siRNA mediated protein knockdown. The alginate substrates with the highest RGD density had the highest levels of siRNA mediated knockdown, as apparent from the increase in siRNA efficacy for cells grown on the DS 16 alginate gels compared to the DS 2 alginate gels both with 133 kPa elastic modulus, as seen in Figure 6.

This difference in siRNA silencing efficacy is unlikely to be due to changes in the ratio of cell number to siRNA concentration, because the day one post-seeding density of U251 cells on the DS 2 alginate gels did not significantly differ from that on the DS 16 alginate, as seen in Figure 5. In addition, differences in cell surface area or morphology were not observed between the DS 2 and DS 16 gels, as seen in Figures 3 and 4, so it is unlikely that these factors accounted for the different RGD density mediated protein silencing effects observed.

In contrast to the findings for alginate gels, there was a statistically significant increase in both cell surface area and knockdown efficacy for cells grown on the TCPS substrate as compared to alginate substrates, as seen in Figure 6. The finding that normalization of the knockdown percentage for TCPS and the different alginate substrates by cell surface area one day after seeding eliminated the difference in siRNA knockdown efficacy between TCPS and some, but not all, of alginate substrates suggests a correlation between changes in cell surface area and knockdown efficacy of siRNA. However, the fact that even after normalization for surface area, there was still a significantly increased siRNA knockdown efficacy for cells grown on the TCPS substrates and some of the alginate substrates suggests that surface area alone does not account for all of the difference in substrate effects.

Quantification of siRNA uptake on different alginate substrates

Further investigation into possible mechanisms of the increased siRNA knockdown efficacy was pursued by evaluating differences in siRNA uptake for cells grown on the different alginate substrates. The cells grown on alginate substrates with the highest RGD density demonstrated the highest siRNA uptake. In addition, normalizing for siRNA uptake eliminated the significant difference in siRNA silencing efficacy seen between the 133 kPa gels of either DS 2 or DS 16. This result suggests a correlation between increased siRNA uptake, increased siRNA silencing efficacy and increased RGD density of the substrate. However, this result does

not distinguish which part of the endocytotic pathway is upregulated. Endocytosis is a multi-step process involving a variety of steps including formation of vesicles, trafficking of intracellular substances and release of cargo from vesicles[50]. More research is required to identify which exact uptake steps are affected by the increased substrate RGD density. Furthermore, these results do not preclude other mechanisms by which RGD density may modulate siRNA knockdown efficacy. Other possibilities include RGD density mediated changes in endogenous siRNA silencing protein effector complexes and/or increased intracellular siRNA stability.

CONCLUSIONS

The findings of this thesis demonstrate that adhesion substrate properties can alter the efficacy of exogenously delivered siRNA. Human glioma U251 cells were successfully grown on alginate hydrogels of varying elastic moduli and RGD density without qualitative difference in morphology or quantitative differences in surface area or cell number. However, increasing the RGD density of alginate hydrogel substrates was able to increase the efficacy of siRNA knockdown at a 33nM concentration of siRNA. Alterations in elastic moduli of the alginate gels over the 53-133 kPa range tested did not alter siRNA efficacy. A lower siRNA concentration of 8.25 nM did not induce siRNA mediated protein knockdown for any of the alginate adhesion substrates tested, despite the ability of this concentration to cause knockdown for cells grown on TCPS. Furthermore, cells grown on TCPS responded with more siRNA mediated protein knockdown than any of the experiments involving alginate substrates. Increases in siRNA uptake were correlated with increased siRNA-mediated protein knockdown for both TCPS and alginate substrates. Overall, the results of this work indicate that adhesion substrate properties can alter the cellular response to siRNA. This has implications for pre-clinical testing of siRNA-based therapeutics in which cells are often grown on a variety of substrates that may be different than the extracellular environment of the *in vivo* target cell.

SUGGESTIONS FOR FUTURE WORK

The findings presented in this thesis provide several avenues for future investigation.

Two broad areas for further work include assessing the generalizability of adhesion substrate control and mechanistic investigation into siRNA delivery efficacy. Each of these directions will be discussed below with suggestions for continued experimental approaches.

Exploring the generalizability of adhesion substrate control of siRNA delivery efficacy

The presented results demonstrate that alteration of RGD density on alginate substrates can modify the efficiency at which siRNA can silence an exogenously introduced gene. One of the limitations of the current work is its use of a single siRNA delivery agent, a single gene target, a single cell type and two types of adhesion substrates. Further work is needed to determine if the findings presented here are generalizable effects across cell type, siRNA gene target, siRNA delivery vector composition and adhesion substrate.

Exploration in different *in vitro* models would be able to determine whether the results presented here are a special case scenario, or instead, broadly representative of adhesion substrates' role in controlling siRNA efficacy. Testing the role of cell type is straightforward. Extensive research has been performed with myocytes and pre-osteocytes on alginate substrates. Thus, one could easily grow either cell type on the range of alginate substrates described here, and expose them to siRNA designed to silence a particular gene product. It would be interesting and useful to compare the response characteristics of neoplastic cell lines, like the U251 cells used in this work, with non-neoplastic cells lines. Difference in responses could have implications for the pre-clinical testing of siRNA, since siRNAs are being considered as possible anti-cancer therapeutics[15] [18][51][52]. A better understanding of the adhesion substrate response of neoplastic cells to siRNA may lead to the development of more realistic *in vitro* models for anti-cancer siRNA therapeutic screening.

In addition, the current work was limited by testing the knockdown of only a single gene, eGFP, chosen for the ease of measuring expression knockdown. Further work could expand the siRNA targeted genes beyond exogenous markers, like luciferase or GFP, to other more physiologically relevant gene products. Depending on the gene silenced, various methods could be used to quantify siRNA mediated knockdown on the different substrates including qRT-PCR or Western blotting.

U251 cells did not show changes in siRNA knockdown efficacy over the range of elastic moduli tested in these experiments. It would be informative to expand the range of alginate stiffness tested to determine if U251 cells are just less responsive to their extracellular environment or if they respond to a different range of stiffness than tested in these experiments. Also, it would be useful to determine whether the results regarding RGD density hold true for substrates other than alginate. Other adhesion substrate hydrogels like PEG, that are both normally non-cell adherent could be controllably functionalized with RGD to see if they yielded similar increased siRNA knockdown efficacy for increases in RGD density[34]. This would allow for further explorations of the adhesion substrates' role in siRNA delivery efficacy, outside of the parameters tested in this work.

Finally, although Lipofectamine 2000 was chosen as a delivery agent for this work due to its widespread use in the laboratory setting, its high cytotoxicity limits its use in clinical applications[53][54]. There are a wide variety of other siRNA delivery agents in current development including polymers, peptides and lipids[20][22][55][56][57]. It would be useful to determine if the increased siRNA knockdown efficacy observed for the Lipofectamine 2000 complexed siRNA also occurred for other modes of siRNA delivery.

Expanding on the current investigation of the effects of growth substrates into the three dimensional environment

The major motivation for the current work is to improve our understanding of how the microenvironment of cells can alter their response to siRNA, in order to determine if investigators should be considering cell adhesion substrate when choosing appropriate pre-clinical models for evaluation of siRNA as therapeutic. However, the use of two dimensional adhesion substrates is an approximation of cell growth *in vivo*, as most clinically relevant cell types normally grow within a three dimensional microenvironment.

Much recent work has shown that cell behavior can be altered by a more physiological three dimensional microenvironment, as opposed to the traditional two dimensional tissue culture substrates[58][59]. Thus, it would be useful to extend the work done here in a two dimensional microenvironment exploring the role of RGD density and stiffness to a three dimensional culture. This could move the applicability of the findings here to the most current in vitro models for pre-clinical therapeutic testing. Alginate would be well suited to this application because it has been previously used for growth of cells in three dimensional gel environments[45][60].

Mechanistic investigation into the role of adhesion substrate control of siRNA efficacy

From the experiments undertaken, it appears that increased siRNA uptake may play a role in the increased siRNA knockdown efficacy seen for alginate gels with higher RGD density. However, it would be interesting to determine whether the increased siRNA uptake is reflective of increased internalization of other compounds. In addition, it remains unclear whether the increased uptake alone is responsible for modulating the siRNA knockdown effect or whether there may also be altered activity of the endogenous siRNA silencing protein complexes.

Although some uncertainty exists regarding the exact mechanism by which siRNA complexed to cationic lipids like Lipofectamine2000 enter cells, most agree that the process is

likely endocytosis dependent. In this work, we observed increased internalization of fluorescently labeled siRNA complexed to Lipofectamine2000 in cells adherent to high RGD density alginate hydrogels. These results lead to the question of whether adhesion substrates can have more broad effects of endocytosis, not simply affecting siRNA complex uptake, but the uptake of other compounds. Specifically, since it appears that siRNA uptake is enhanced by certain adhesion substrates, it would be of interest to determine whether this increased uptake extends to other molecules. Although previous studies have mostly focused on linking adhesion substrate increased proliferation with increased delivery of biomacromolecules, it is possible that increased rates of endocytosis may also be an important factor in adhesion substrate control of biomacromolecule delivery. Although the study of endocytosis is a complex and rapidly evolving field, systematic experimentation to assess endocytotic uptake of different ligands in response to variations in adhesion substrate may yield results that provide useful frameworks in which to understand the tripartate interaction between cells, their adhesion substrates and endocytosed ligands. Experiments might employ canonical ligands known to be endocytosed by specific pathways, e.g. transferrin in the clathrin dependent pathway or Lactosylceramine in the caveolin dependent pathway[61][62].

It is also possible that the increase in siRNA efficacy seen on the high RGD density alginate adhesion substrates is also influenced by increased activity of the siRNA silencing pathway. As discussed in the introduction, a variety of different enzymes are required for siRNA guided destruction of mRNA. Perhaps changing the adhesion substrate can upregulate protein effectors involved in this pathway. To test this hypothesis, it may be valuable to quantify the amount of siRNA effector enzyme (e.g. RISC) mRNA through PCR or proteins through

Western Blotting. Increases in the siRNA effector enzymes would indicate an increased capacity for enacting siRNA mediated protein silencing within the cell.

Overall, the presented results within this thesis are provoking, with significant implications for preclinical testing of siRNA. The body presents many examples of cells with altered extracellular environments, and application of siRNA as a therapeutic may require optimization with regard to cellular adhesion substrate. Furthermore, increasing the successful translation of siRNA therapeutics from the bench to the bedside will only be enhanced by elucidation of factors affecting siRNA delivery. Understanding the generalizability of the observed results, as well as the mechanism by which they occur, is critical for the long-term importance of the presented findings.

SUMMARY

Short interfering RNAs (siRNAs) are an extremely promising class of therapeutics due to their ability to prevent the synthesis of disease causing proteins. In order to mediate their useful effects, siRNAs must be delivered to the inside of cells. However, delivering enough siRNA intracellularly to yield clinically relevant effects poses a major practical challenge. The presented work aims to address this barrier through an enhanced understanding of the factors that affect siRNA behavior in cells. Specifically, it explores the hypothesis that the surface on which cells adhere modulates the efficiency of administered siRNA to cause protein synthesis inhibition. To investigate this hypothesis, cells were grown on materials that were systematically varied in properties altering cell-surface adhesion. Increased siRNA efficiency was observed when cells were grown on materials designed to increase the extent of cell adhesion. These results demonstrate that the surface on which cells adhere can change cellular response to siRNA and introduce an important new variable for consideration in the design of siRNA therapeutics.

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TABLES AND FIGURES

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Table 1. Four types of alginate hydrogels were synthesized with two different values of RGD density and elastic modulus. DS refers to degree of substitution or the approximate number of RGD ligands per alginate polymer. This material is reproduced with permission of John Wiley & Sons, Inc.

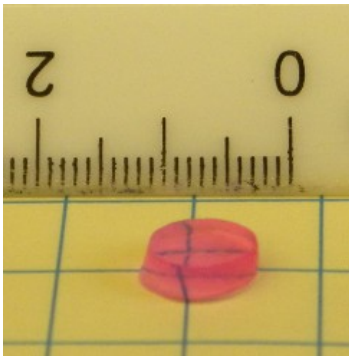


Figure 1. Photograph demonstrating the typical appearance of an alginate hydrogel. Scale included in photograph corresponds to a length of 2 cm.

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Figure 2. (A) The mean elastic modules of 2% by weight alignate hydrogels with 50 mM (□) and 100 mM (■) of calcium crosslinker was measured during a four day incubation period with three experiments per value shown. Error bars represent the standard deviation of the three experiments. Gels synthesized with 50 mM and 100 mM Ca^{++} had elastic moduli of $53 \pm 23\text{kPa}$ and $133 \pm 31\text{ kPa}$, respectively, after four days of incubation. (B) There was no significant difference in mechanical properties of alginate hydrogels without and with a high degree of RGD modification. Hydrogels were formed from 2.0% weight percent alginate and a constant calcium cross-linker concentration of 50 mM (53 kPa elastic modulus). The alginate was either without RGD modification (light gray bars) or with DS16 RGD modification (dark gray bars). Values

shown are the mean and error bars are the standard deviation of three experiments. There were no statistically significant differences between the mean elastic values of alginate hydrogels with and without RGD modification. This material is reproduced with permission of John Wiley & Sons, Inc.

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Figure 3. Fluorescent imaging of eGFP expressing U251 cells adherent to substrates of varying RGD degree of substitution and varying stiffness at 1 day after seeding. Bar represents 100 μm . This material is reproduced with permission of John Wiley & Sons, Inc.

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Figure 4. Mean surface area measurements of cells adherent to different types of alginate substrates over time. Surface area shown for U251 cells grown on 53 kPa DS 2 alginate gels (black), 133kPa DS 2 alginate gels (dark gray), 53 kPa DS16 alginate gels (light gray), 133 kPa DS 16 alginate gels (white) and TCPS (striped) at one and four days after seeding. Mean surface area measurements were calculated from the individual surface area measurements for cells visible in three 0.58 mm² fields on each gel. Error bars represent the standard deviation among average cellular area of each 0.58 mm² field. This material is reproduced with permission of John Wiley & Sons, Inc.

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Figure 5. Number of U251 cells present on alginate substrates of varying RGD density and elastic moduli over four days. (A) Number of adherent cells growing on 53 kPa DS 2 alginate gels (black), 133 kPa DS 2 alginate gels (dark gray), 53 kPa DS16 alginate gels (light gray), 133

kPa DS 16 alginate gels (white) and TCPS (striped) at either one, two or four days post seeding. (B) Data from part A normalized to number of cells present adherent to each gel at day 1 after seeding. Values displayed are the mean cell number and error bars represent the standard deviation for four randomly chosen 0.58 mm² fields. This material is reproduced with permission of John Wiley & Sons, Inc.

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Figure 6. Relative eGFP fluorescence expression in U251 cells grown on alginate adhesion substrates differing in RGD density (DS or degree of substitution) and mechanical stiffness after application of 8.25nM (light gray) and 33 nM (dark gray) of siRNA. eGFP expression was quantified via FACS on more than 50 cells per treatment three days after siRNA application. Each knockdown percentage shown represents the mean of three replicate experiments. Error bars represent the standard deviation. Asterisks indicate statistical significant differences ($p < 0.05$) via a student t test either for relationships indicated via a line or in comparison to all other treatments of the same siRNA concentration. This material is reproduced with permission of John Wiley & Sons, Inc.

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Figure 7. Verification that siRNA results in specific targeting of eGFP expression knockdown. Normalized fluorescence intensity is shown for stably eGFP expressing clonal populations exposed to a sequence specific siRNA (light gray) to a scrambled sequence control siRNA (dark gray). Normalized fluorescence intensity was measured as a ratio between the green fluorescence intensity of the cell population when compared to an untreated control. The above

results represent the mean population fluorescent values for a single experiment. This material is reproduced with permission of John Wiley & Sons, Inc.

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Figure 8. (A) Fluorescently labeled siRNA uptake for cells grown on different alginate substrates normalized by comparison to a non-siRNA treated control. U251 cells were incubated with 50 nM AF488 labeled siRNA for 4 hours before fluorescence uptake was determined by FACS analysis on at least 50 cells per treatment. Shown values are the mean normalized fluorescence and error bars represent the standard deviations of three replicate experiments. Asterisks indicate significance ($p < 0.05$) via students t-test for comparisons indicated by a line or in comparison to all other treatments. (B) eGFP expression in U251 cells after exposure to 8.25 nM (light gray) or 33 nM (dark gray) anti-eGFP siRNA on alginate adhesion substrates after normalization for fold increase in mean fluorescent intensity at 488 nm 1 day after seeding. FACS was used to determine the shown mean values of fluorescence intensity 3 days after siRNA application for at least 50 cells per treatment and three replicate experiments. Error bars represent the standard deviation for the three replicate experiments. ANOVA analysis with post-hoc tests showed no significant difference in knockdown percentage among cells grown on the different growth substrates for the same siRNA concentration after normalization for siRNA uptake. This material is reproduced with permission of John Wiley & Sons, Inc.